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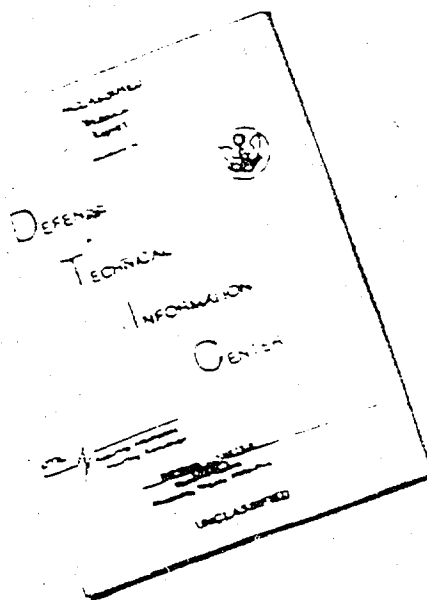
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TECHNICAL MANUSCRIPT 17

LARGE-SCALE PRODUCTION
OF THE PROTECTIVE ANTIGEN
OF BACILLUS ANTHRACIS
IN ANAEROBIC CULTURES

APRIL 1963

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UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

NOTES

U.S. ARMY CHEMICAL-BIOLOGICAL-RADIOLOGICAL AGENCY
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Fort Detrick, Frederick, Maryland

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Milton Puzias
Lee C. Manning
Joe W. Lynch Jr.
Eugene S. Barclay
Ira Abelow
George G. Wright

Medical Investigation Division
DIRECTOR OF MEDICAL RESEARCH

Project JA012501802805

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ABSTRACT

A production-proving test is described for the preparation, by the anaerobic culture method, of large volumes of culture filtrate containing immunologically potent protective antigen of B. anthracis. The process consisted of the anaerobic culture, in a chemically defined medium, of a selected production strain. The culture was then clarified and sterilized by filtration through sintered-glass filters. The sterile culture filtrate was adsorbed onto a preformed aluminum hydroxide gel and the stabilized gel-antigen complex was concentrated. The final product had high immunizing potency, as shown by both in vivo and in vitro assays, and was well tolerated in man. Stability of the product to accelerated aging was good, and storage at 4°C for one year caused only a minor loss in protective activity. Large volumes of the highly antigenic gel-adsorbed protective antigen were readily produced by the method described.

CONTENTS

Acknowledgments	3
Abstract	3
I. INTRODUCTION	5
II. MATERIALS AND METHODS	5
A. Cultures	5
B. Growth Medium	5
C. Production Equipment	7
D. Aluminum Hydroxide Gel	7
E. Assay Methods	9
F. Process Description	9
G. Filtration and Adsorption	9
H. Final Processing	10
III. RESULTS	10
IV. DISCUSSION	15
Literature Cited	17

FIGURES

1. The Steam-Jacketed Fermentation Tank and Controls	8
2. The Chem-Flow Filter Assembly	8
3. Relationship of Complement Fixation Titer, Bacterial Count, Glucose Utilization, and pH Change in Cultures of <u>B. anthracis</u>	12
4. Stability of the Gel-Adsorbed Product After Heating at 37°C	14

TABLES

I. Constituents Required for 300 Liters of 1095 Medium	6
II. Antigenicity Obtained in Six Successive 300-Liter Cultures	11
III. Antigenicity of Gel-Adsorbed Antigen after Storage at 4°C For One Year	13

I. INTRODUCTION

The protective antigen elaborated by Bacillus anthracis during aerobic growth has been shown to be effective in immunizing animals and man against anthrax.¹⁻³ Subsequent research established that protective antigen was also elaborated in good yield under anaerobic conditions in chemically defined media.⁴ Adsorption of the culture filtrate antigen onto a pre-formed aluminum hydroxide gel and preservation and stabilization of the adsorbed product was described by Puziss and Wright.⁵ The stable final product had high antigenicity in experimental animals and was well tolerated in man. These studies indicated that the anaerobic process could be readily adapted to production of antigen on a practical scale. The objective of the work presented in this report was a study of the requirements for adaptation of the anaerobic culture method to provide a large-scale process for production of anthrax protective antigen.

II. MATERIALS AND METHODS

This work was performed primarily at the West Point, Pa., facilities of the Merck, Sharp and Dohme Division of Merck & Co., Inc. Two of the authors (Lee G. Manning and Eugene S. Barclay) are on the staff there. All animal immunization and challenge studies were performed at Fort Detrick.*

A. CULTURES

Strain V770-NP1-R, a nonencapsulated, nonproteolytic, and avirulent mutant of B. anthracis, was used as inoculum.⁶ Immunized animals were challenged with the virulent Vollum strain of B. anthracis.⁶

B. GROWTH MEDIUM

The chemically defined 1095 medium described by Wright et al⁴ was used; it was made up as a 25-fold concentrated stock and diluted to required volume in the fermentation tank. The chemicals were of C.P. or reagent grade; all water was freshly distilled and obtained from the plant service line at 180°F. Composition of the defined medium is presented in Table I.

* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

TABLE 1. CONSTITUENTS REQUIRED FOR 300 LITERS OF 1095 MEDIUM

CONSTITUENT	CONCENTRATION Grams/12 liters	CONSTITUENT	CONCENTRATION Grams/12 liters
<u>AB-G stock</u>		<u>C stock</u>	
Biotin, crystalline	0.15	KH_2PO_4	204
Thiamine · HCl	0.12	K_2HPO_4	251
Adenosine	0.30		
L-Alanine	2.70		
L-Tryptophan	3.12		
DL-Serine	6.24	<u>Ferrous sulfate</u>	
L-Arginine · HCl	6.24	<u>solution b/</u>	<u>Amt/3 liters</u>
L-Proline	8.76	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.87 gram
Glycine	8.76	Concd HCl	4.5 ml
DL-Methionine	9.00	Heat to dissolve.	
DL-Threonine (allo-free)	18.00	<u>Pyridoxal solution b/</u>	<u>Gram/600 ml</u>
DL-Valine	18.00	Pyridoxal · HCl	0.3
DL-Aspartic acid	19.20		
DL-Isoleucine	19.20	<u>Sodium bicarbonate a/</u>	
DL-Phenylalanine	20.40	<u>solution</u>	<u>Gram/6 liters</u>
L-Histidine · HCl	28.80	NaHCO_3	750
DL-Leucine	38.40		
L-Glutamic acid	30.40		
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1.20		
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.00		
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	4.44		
Glucose	300		
Guanine · HCl	2.76		

- Boil to dissolve in 600 ml H_2O plus 9.0 ml concentrated HCl, add to AB stock, and bring final volume to 12 liters.
- Sterilized by filtration.
- Sterilized by autoclaving for 45 minutes at 121°C .

C. PRODUCTION EQUIPMENT

A glass-lined, jacketed, closed tank of 150-gallon capacity (special type M) ^a served for preparation of medium and growth of the culture. This vessel (Figure 1) was equipped with a variable-speed impeller to agitate the contents, and with the customary temperature and motor controls. Cultures were filtered through a combination of sintered-glass ^b clarifier and sterilizer units, each of one squarefoot area. The clarifier filter (Five-micron porosity) was connected in series with two one-micron-porosity sterilizing filters. A flow rate of about 0.82 liter per minute was achieved; this required back-flushing of the clarifier filter with sterile water once an hour. A standby 5-micron-porosity clarifier unit was used during the back-wash period. The back-flushed fluid was collected in a carboy, autoclaved, and discarded. The filtration arrangement is shown in Figure 2. The clarifier filters (a) are attached to the sterilizer filters (b) arranged in series. Culture from the fermentor enters the assembly from the tube at right (c); sterile filtrate leaves in the tube at left through an opening in the wall.

Sterile culture filtrate was passed into a second closed, glass-lined, jacketed tank (E.S. series) ^c for adsorption and holding at 4°C. This tank was also equipped with an impeller and the customary refrigerant and motor controls. The gel-antigen complex was processed in an International Centrifuge, Model 13L. ^d

D. ALUMINUM HYDROXIDE GEL

The gel used as adsorbent and adjuvant for the antigen was prepared by the method of Hansen⁷ in a glass-lined, steam-jacketed, open tank equipped with stirring paddles. A continuous-flow nozzle-discharge centrifuge ^a was used to remove and wash the gel. The final gel product was tested for its antigen-adsorbing ability with a culture filtrate of known immunizing activity. Standard gel of proved antigen-adsorbing capacity, kindly supplied by Dr. Inga Scheibel of the Statens Serum Institut, Copenhagen, Denmark, was used as an adsorbent control.⁶ The gel product used in the present work resembled the standard Danish gel in its adsorptive capacity for antigen.

-
- a. The Pfaudler Mfg. Co., Rochester, N. Y.
 - b. The Chem-Flow Corp., Little Falls, N. J.
 - c. International Equipment Co., Boston, Mass.
 - d. The Sharples Corp., Philadelphia, Pa.

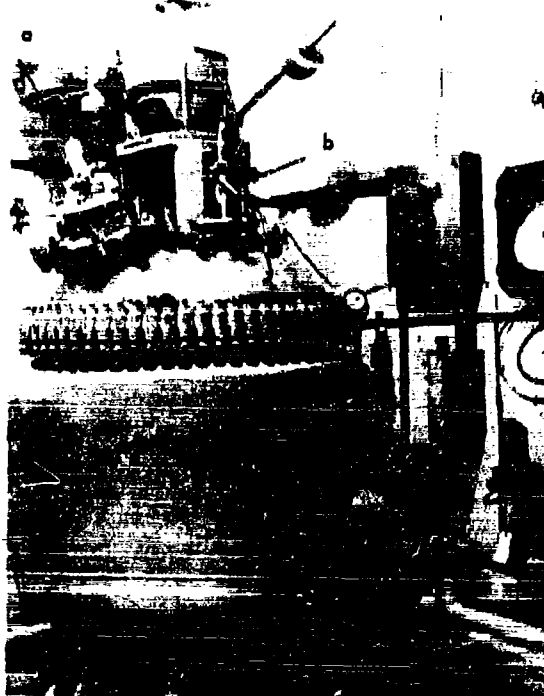


Figure 1. The Steam-jacketed Fermentation Tank and Controls.
(FD Neg. C-6951)

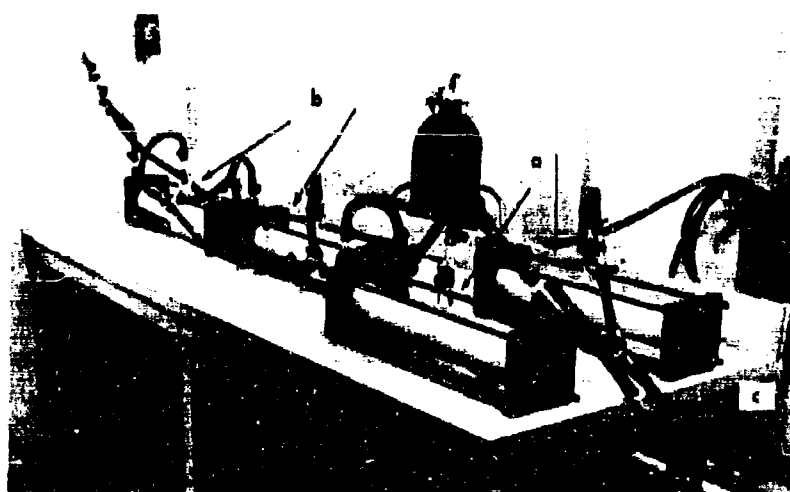


Figure 2. The Chem-Flow Filter Assembly. (FD Neg. C-6952)

E. ASSAY METHODS

Culture filtrates were tested for in vitro antigen activity by the complement fixation method of McGann, Stearns, and Wright⁸ and also by the agar gel diffusion method of Thorne and Belton.⁹ In vivo immunizing activity was assayed by immunization and challenge of rabbits.⁸ All animals received a single 0.5-ml subcutaneous immunizing dose of antigen diluted in saline, as shown in the Tables. Two weeks later the animals and unimmunized controls were challenged intracutaneously with 10,000 spores of the challenge strain in a volume of 0.25 ml. This dose represented 500 to 1000 LD₅₀. Residual glucose in the culture was determined by the anthrone method of Morris, as described by Naish.¹⁰ The bacterial counts were estimated turbidimetrically in a colorimeter against a nephelometric standard suspension of Salmonella typhosa obtained from the Division of Biological Standards, National Institutes of Health. Aluminum, aluminum oxide concentration, and formalin content in the final antigen product were determined by the methods described in U. S. Pharmacopeia XVI.¹¹ Sterility and safety tests performed during production, as well as on the final product were in accordance with Public Health Service Regulations.¹²

F. PROCESS DESCRIPTION: THE MEDIUM

AB-G and C stocks, 12 liters each, were added to 266.3 liters of distilled water contained in the 150-gallon fermentation tank. The tank and its contents were sterilized by heating to 120°C and cooling immediately to 37°C. After the temperature had dropped to 100°C, pressure was equilibrated by allowing air to enter through a sterile air filter; six liters of sterile sodium bicarbonate (12 per cent) were then added. Sterile ferrous sulfate and pyridoxal hydrochloride solutions, pooled in a 20-liter Pyrex glass bottle, were added to the contents of the tank and the tank was inoculated with the seed culture. Final pH of the medium was about 7.9. A sterile tube, inserted through the inoculating valve, was used to introduce nitrogen (sterilized by a filter) into the tank to a pressure of 5 psig. The gas was introduced close to the surface of the medium for 25 minutes and displaced the air through the top valve of the tank; the valve was then closed.

The culture was incubated at 36° to 38°C under nitrogen for approximately 27 hours, with agitation at a constant rate of 20 revolutions per minute. Samples for the various tests were removed through a sterile tube at selected intervals during growth.

G. FILTRATION AND ADSORPTION

At the termination of incubation, the pH of the culture, which had dropped to pH 6.9, was raised to pH 8.0 with sterile 2N sodium hydroxide and the culture was cooled to 18° to 20°C. A pressure of about 10 psig of nitrogen was applied, and the culture was forced through a glass valve in the bottom of the tank, through the clarifying and sterilizing filters, and into the

holding tank. Approximately six hours was required to filter the 300 liters of culture. Twelve liters of the sterile aluminum hydroxide gel at a temperature of 22°C was added to the filtrate. The mixture was agitated for 30 seconds at 60 revolutions per minute and then cooled to 4°C. Three times daily for five days, the suspension was agitated for 30 seconds. An alternate method, preferable when proper equipment is available, would be to agitate continuously at 15 to 20 revolutions per minute for two days.⁵ Agitation was discontinued 20 hours prior to removal of adsorbed antigen from the tank to allow the material to settle. Approximately 31 liters of the concentrated suspension of adsorbed antigen was removed through the bottom valve. The supernatant was discarded.

H. FINAL PROCESSING

The adsorbed antigen was distributed aseptically into sterile 1.5-liter Pyrex glass centrifuge bottles, each containing several sterile glass beads. A centrifugal force of 780 x g was applied for 10 minutes at 4°C, and the supernatant was removed aseptically by vacuum from each bottle and discarded. The gel slurry remaining was strained into a sterile 43-liter Pyrex glass carboy through a sterile No. 62 mesh nylon strainer. The pooled slurry (approximately 10 liters) was resuspended in cold, sterile, physiological saline solution to 28.5 liters. Preservatives were added and the carboy was held at 4°C until potency and safety tests were completed. The preservative consisted of 750 ml of a 1:1000 solution of recrystallized benzethonium chloride* to a 1:40,000 final concentration. As a stabilizer, 750 ml of one per cent formalin was added to a final concentration of 0.0092 per cent formaldehyde. The final volume of product was 30 liters; this represented a tenfold concentration from the original 300 liters of culture filtrate. Determinations of sterility by cultural tests, safety tests with mice and guinea pigs, tests for antigenicity in rabbits, and tests for aluminum and formalin content were performed. Finally, vials were filled with 20-ml quantities of the antigen. Approximately 60,000 doses were obtained from the 300-liter lot.

III. RESULTS

In the early phases of the investigation the fermentation tank containing the culture medium was inoculated with 500 spores per milliliter of the production strain of *B. anthracis*, as described previously.⁶ Under these conditions the initiation of growth was delayed, maximum turbidity and utilization of glucose were reduced, and only small amounts of protective antigen were detected in culture filtrates.

* Phamarol, produced by Parks, Davis & Co.

The difficulty was overcome by seeding the tank with an actively growing vegetative inoculum rather than with the spore suspension. Ten liters of the culture medium in a carboy was inoculated with 10^7 spores of strain V770-NPl-R, incubated statically in air for 26 hours at 37°C, and added to the main lot of medium. Under these conditions good growth and maximum accumulation of antigen occurred in the 300-liter culture after incubation for approximately 26 hours. At intervals during the incubation period determinations were carried out of the pH, the bacterial count, the complement fixation titer, and the residual glucose; data compiled from several production experiments are shown in Figure 3. Glucose utilization was the most sensitive and useful indicator of the course of the fermentation; earlier studies had shown that maximum antigen accumulation coincided with the point at which glucose in the medium approached exhaustion. After 18 to 20 hours of growth it was possible to extrapolate the glucose utilization curve to estimate the time at which antigen elaboration would be at a maximum, and filtration of culture could be initiated. Antigenicity of six consecutive production lots as measured by *in vivo* and *in vitro* assays is presented in Table II.

TABLE II. ANTIGENICITY OBTAINED IN SIX SUCCESSIVE
300-LITER CULTURES

Lot	TITERS OF CULTURE FILTRATE		ANTIGENICITY OF GEL-ADSORBED PRODUCT ^a			
	Complement fixation 50% Units/ml	Gel Diffusion Endpoint	Antigen Dilution			
			1:10	1:30	1:90	Controls
5	160	1:4	4/8; 6/8			0/2
6	160	1:4	6/8			0/2
7	160	1:4	4/8; 4/8			0/2
8	160	1:2	7/8; 7/8	5/8	5/8	0/2
9	160	1:2	7/8; 8/8	7/8	7/8	0/2
10	160	1:2	6/8			0/2

a. The antigen product was diluted with saline as shown; a single 0.5-ml immunizing injection was given. Ratios refer to surviving rabbits over the total number challenged.

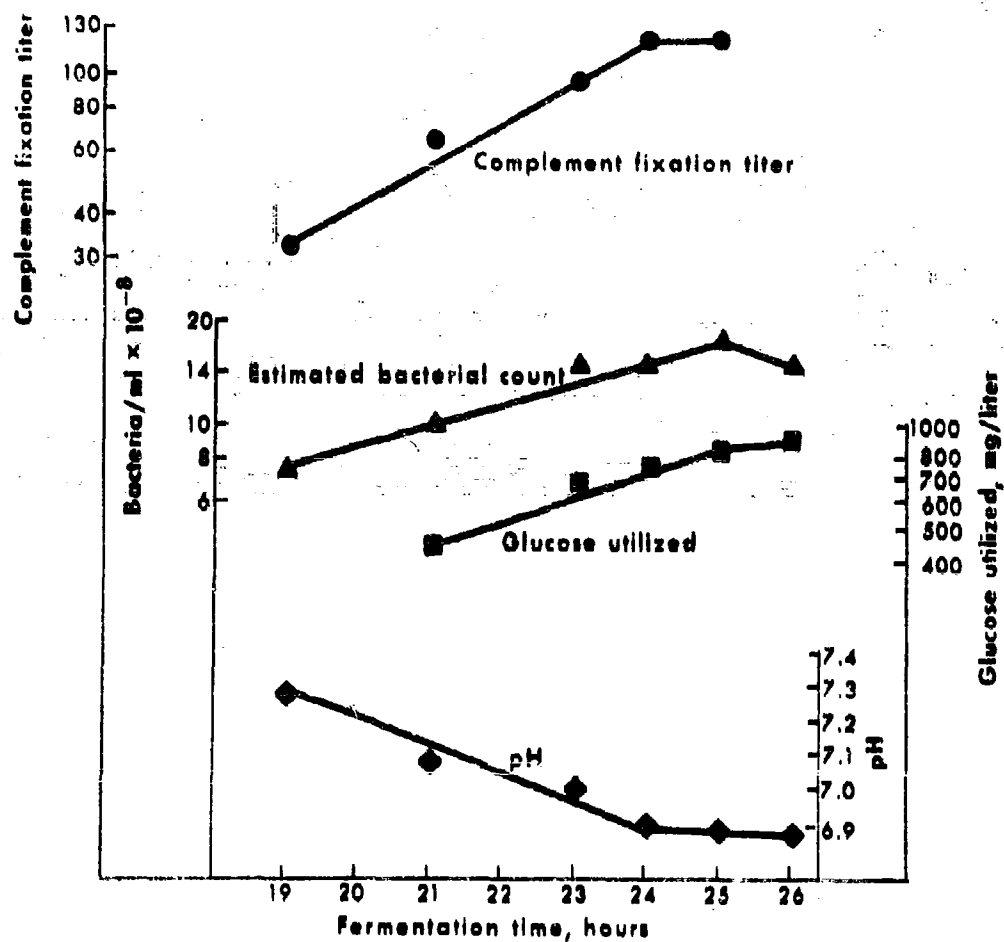


Figure 3. Relationship of Complement Fixation Titer, Bacterial Count, Glucose Utilization, and pH Change in Cultures of *B. anthracis*.

Stability to accelerated aging at 37°C of the gel-adsorbed product was studied. Lot 9 antigen was incubated at 37°C and assayed in rabbits at weekly intervals to determine the loss of immunogenic potency. The results indicated that the antigen had considerable stability to accelerated aging; approximately 60 per cent of the test animals survived challenge after immunization with antigen heated at 37°C for eight weeks (Figure 4). These results indicated that stability to accelerated aging of the product was equivalent to that of an experimental gel-adsorbed antigen prepared previously in the laboratory.⁶ Several lots of antigen were re-assayed after storage for one year at 4°C. Results of these re-assays indicated that antigenicity of the stored product decreased to a slight but minor extent in some of the lots (Table III).

TABLE III. ANTIGENICITY OF GEL-ADSORBED ANTIGEN
AFTER STORAGE AT 4°C FOR ONE YEAR

Lot Number	Survival Ratio ^a			Controls
	Antigen dilution 1:10	1:30	1:90	
8	5/6	---	3/6	0/2
9	5/6	4/6	3/6	0/2
10	8/8	---	6/8	0/2

a. The antigen product was diluted with saline as shown; a single 0.5-ml immunizing injection was given. Ratios refer to surviving rabbits over the total number challenged.

Several lots of gel-adsorbed antigen have been used in a continuing program of immunization of personnel occupationally exposed to anthrax, either in research laboratories or in industry. The antigen was well tolerated; mild and transitory local reactions were elicited at the site of injection in a very small percentage of those immunized. Further studies on the antigenicity of the product are in progress.

IV. DISCUSSION

The study achieved its objective, the development of a laboratory method into a process suitable for large-scale production of the protective antigen of *B. anthracis*. The process described here appears to be a reliable method for producing large volumes of the antigen in a form suitable for immunization of man; it is adaptable to a further increase in the scale of production should the need exist. Replacement of the spore inoculum with an actively growing vegetative inoculum was the only change that was required to adapt the laboratory procedure to use with 300-liter lots of culture in tanks. Substitution of the vegetative inoculum not only led to satisfactory growth and elaboration of antigen, but also reduced the incubation time of the tank culture to approximately 26 hours. No explanation was established for failure of the tank-scale cultures to grow as satisfactorily as the laboratory-scale cultures when a spore inoculum was used.

The preparations of antigen produced in the present study appear to be at least equivalent to those prepared in the laboratory in all respects tested. Complement fixation titers of the culture filtrates and the antigenicity of the adsorbed products in rabbits were generally similar. Gel diffusion titers of the present filtrates were not significantly different from unpublished titers obtained with laboratory lots of filtrate; no valid comparison can be made with diffusion titers recorded by others because of the use of different antisera and the probable influence of minor variations in technique. The present preparations also resembled laboratory preparations in stability during storage at 4°C and during accelerated aging at 37°C.

The least satisfactory portion of the over-all process was the centrifugation of the gel-adsorbed product. This procedure is time-consuming and affords a potential source of contamination of the product. Newer concepts in the production method are under consideration in an effort to eliminate the troublesome centrifugation and maintain an essentially closed production system.

The protective antigen is a rather labile substance elaborated only under carefully controlled conditions. The satisfactory results obtained in production of this substance in 300-liter lots may justify attempts to produce other labile biologicals on a large scale by bioengineering techniques.